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SlyA regulates the collagenase-mediated cytopathic phenotype in multiresistant *Salmonella*☆

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Abstract

Salmonella enterica serotype Typhimurium phagetype DT104 (DT104) is a foodborne pathogen with a multiresistant phenotype conferred by a genomic-based integron structure designated as SGI1. Recently, a novel cytopathic phenotype was ascribed to several isolates of DT104 recovered from veal calves. This phenotype is dependent upon *clg*, a gene encoding a collagenase in *Salmonella*. Using a novel transposon system and an RT-PCR assay for detection of *clg* expression, we identified SlyA as a regulator of the collagenase-mediated phenotype. The function of SlyA, in regards to *clg* expression, is to repress the synthesis of Clg. Derepression ensued in the absence of SlyA or in the presence of a truncated version of SlyA with the latter being relevant for maintenance of another virulence aspect mediated by SlyA, i.e. survival within macrophages. The SlyA-mediated effect on *clg* expression was restricted to DT104 and other *Salmonella* phagetypes and serotypes possessing SGI1 thus suggesting co-regulation by an SGI1-specific component.
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Keywords: *Salmonella*; Antibiotic resistance; Cytopathic; DT104

1. Introduction

Salmonella enterica serotype Typhimurium phagetype DT104 (DT104) is a multiple antibiotic resistant pathogen possessing a chromosomal arrangement of genes, i.e. an integron structure [1] designated as *Salmonella* genomic island 1 (SGI1), that facilitates multiple antibiotic resistance. DT104 additionally can exhibit a hypervirulent phenotype based on increased bovine mortality [2] and human hospitalization [3] rates.

Another DT104 virulence phenotype is related to the expression of a collagenase, encoded by *clg*, in veal calves [4]. Specifically, this collagenase confers a novel cytopathic phenotype as evidenced by pathologic findings uncharacteristic of salmonellosis. These pathologies included abomasitis, peritonitis and polyserositis. Interestingly, the cytopathic phenotype was not exhibited in vitro and thus a heterologous expression system was needed for reconstitution of the phenotype. A repression system apparently regulates *clg* expression and derepression occurs in veal calves although the mechanism is unknown.

Studies herein were undertaken to determine genetic elements involved in repression and derepression of *clg* expression. Transposon mutagenesis was performed in order to disrupt and subsequently identify gene(s) involved in *clg* repression. Insertion mutants were screened for *clg* expression as determined by an RT-PCR assay. RT-PCR positive clones, along with transformants thereof and other relevant strains, were then assessed for the cytopathic phenotype.

2. Results

2.1. Creation of DT104 mutants and derepression of clg expression in two mutants

In our previous studies with the cytopathic DT104, *S. enterica* serotype Typhimurium phagetype DT104 strain

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Table 1
Summary of strains and plasmids used in this study

Strain or plasmid	Description	Reference(s)	Antibiotic resistance[a]	SGI1 presence[b]
LNWI	Cytopathic DT104 strain isolated from veal calves	[4,5]	ASSuT[c]	Yes
LNWIΔSlyA	Deletion of SlyA	This study	ASSuTZ	Yes
LNWI-SlyA′	Truncation of SlyA	This study	ASSuTZ	Yes
DT104 strain 795	DT104 strain chosen at random	[6]	ACSSuTK	Yes
SL1344	Laboratory strain of *S. typhimurium*	[8]	Sensitive	No
TH11	DT104 strain lacking SGI1	[6]	Sensitive	No
S. agona	Serotype possessing SGI1	[9]	ACSSuT	Yes
S. infantis	Serotype possessing SGI1	[6]	ACSSuT	Yes
S. dublin strain 9276	Serotype lacking SGI1	[6]	ACSSuT	No
U302, DT193, and DT120	DT104-related phagetypes	[6]	ACSSuT	Yes
DT208	DT104-related phagetype	[6]	ACSSuT	No
202/37	*S. typhimurium*	[6]	Pan-resistant[d]	No
pAS-*slyA*	Plasmids containing an antisense insert of *slyA*; pCR2.1 and pCRXL are high copy while pBAD is medium copy	This study	K (pCR2.1) or Z (pCRXL) or A (pBAD)	NA
pSlyA	Plasmid containing a sense insert of *slyA*	This study	K	NA
pSlyA	Plasmid containing a sense insert of *slyA′*	This study	K	NA
pBRP	Plasmid encoding the bacteriocin releasing peptide	[7]	C[e]	NA

[a] A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulfamethoxazole; T, tetracycline; K, kanamycin; Z, zeocin.
[b] NA, not applicable.
[c] Strain LNWI possess an SGI1 subtype containing a truncated chloramphenicol resistance gene thus these variants are chloramphenicol sensitive [1,6].
[d] Resistant to most antibiotics except fluoroquinolones and zeocin; transformation with pAS-*slyA* was performed using pCRXL instead of pCR2.1 so that zeocin could be used as a resistance marker.
[e] Not utilized in most DT104 strains, except LNWI, due to SGI1-mediated chloramphenicol resistance.

LNWI (LNWI, [5]) was the model strain (strains are summarized in Table 1). In order to identify genes involved in *clg* expression, we set out to use transposon-based mutagenesis. Unfortunately, LNWI is resistant to most antibiotics that are commonly associated with selection of transductants. Therefore, we developed a novel zeocin (Invitrogen)-based transposon (*TnZeo*) for use in multiple antibiotic resistant *Salmonella*.

Using an RT-PCR assay for *clg* expression [4] and high-throughput screening, two insertional mutants were capable of expressing *clg* (Fig. 1). These mutants were subjected to inverse PCR (primers provided in Table 2), whereby

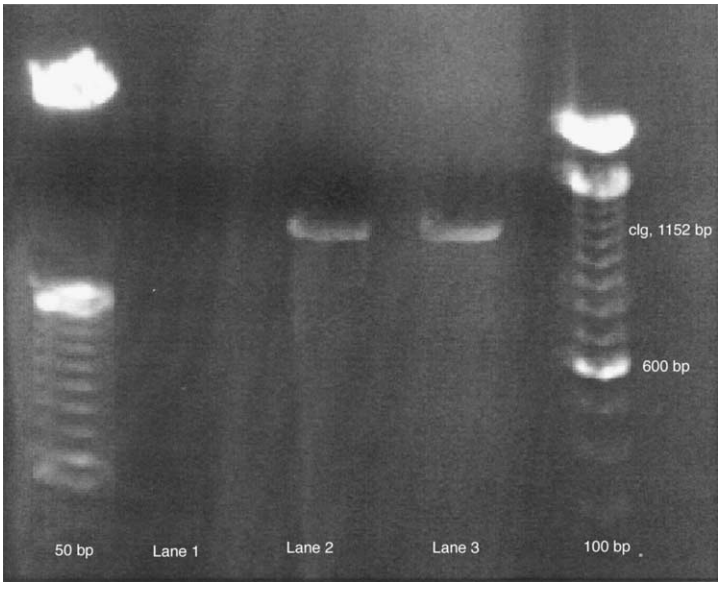

Fig. 1. RT-PCR-based evaluation of *clg* expression in two insertional mutants of LNWI. Lane 1, LNWI; Lane 2, LNWIΔSlyA; Lane 3, LNWI-SlyA′. Molecular weight ladders are shown on the far right (100 bp) and far left (50 bp) lanes with the 600 bp marker indicated on the right. The size of the expected *clg* transcript is also shown on the right. RT-free assays yielded no amplicons (data not shown).

Table 2
Nucleotide sequences and uses of primers described in this study

Primer name	Sequence (5′–3′)	Use
SalZeoF	GTCGACATGGCCAAGTTGACCAGTGCCGT	PCR-based amplification of the zeocin-resistance gene
BamZeoR	GGATTCTCAGTCCTGCTCCTCGGCCACGA	PCR-based amplification of the zeocin-resistance gene
FP-1	ATTCAGGCTGCGCAACTGT	Manufacturer's primer used for amplifying *TnZeo* for creating the EZ::*TnZeo* transposome
RP-1	GTCAGTGAGCGAGGAAGCGGAAG	Manufacturer's primer used for amplifying *TnZeo* for creating the EZ::*TnZeo* transposome
ClgF	ATGCGCCAGCAACCTCACTAT	RT-PCR
ClgR	ACTTTATGCAGGTCGGCAGGCAT	RT-PCR
SlyAF	ATGGAATCGCCACTAGGTTCT	RT-PCR and cloning of *slyA*
SlyAR	ATGAATGACCTCTTCCATCTC	RT-PCR
SlyAstopR	TCAATCGTGAGAATGCAATTCCAT	Cloning of *slyA*
slyA'stopR	TCAGGGTTGAGATGTGTATAAGAGACAG	Cloning of *slyA′*
SqFP	GCCAACGACTACGCACTAGCCAAC	Inverse PCR
SqRP	GAGCCAATATGCGAGAACACCCGAGAA	Inverse PCR

the *TnZeo* insertions were found in *slyA*. As shown in Fig. 2, the insertion in isostrain LNWIΔSlyA leads to a deletion of SlyA, whereas the insertion in isostrain LNWI-SlyA′ leads to a truncation of SlyA.

2.2. Cytopathicity of LNWI, TnZeo insertional mutants of LNWI, and control strains

Assessment of the cytopathic phenotype was performed using a tissue culture assay in which a live-dead stain was used for visually quantitating eukaryotic cell death. LNWI and the two insertional mutants were the principle strains used in these studies. As a control, LNWI was transformed with a high copy plasmid containing an antisense copy of *slyA* (pAS-*slyA*) that yields an excess of antisense transcripts capable of annealing to and thus sequestering the sense transcripts of *slyA*. That is, the sense–antisense RNA duplex is not recognized by the ribosome thereby preventing translation of the sense transcript. Other strains included: both LNWIΔSlyA and LNWI-SlyA′ transformed

∇*TnZeo* insertion site for LNWIΔSlyA

```
ATGGAATCGCCACTAGGTTCTGATCTGGCACGGTTGGTGCGCATTTGGCGTGCTCTGATTGACCAT
M  E  S  P  L  G  S  D  L  A  R  L  V  R  I  W  R  A  L  I  D  H

CGCCTCAAGCCTCTGGAATTGACGCAGACACATTGGGTCACGTTGCACAATATTCATCAATTGCCG
R  L  K  P  L  E  L  T  Q  T  H  W  V  T  L  H  N  I  H  Q  L  P

CCTGACCAGTCGCAGATTCAATTGGCTAAAGCGATAGGCATTGAGCAGCCATCGCTGGTACGCACG
P  D  Q  S  Q  I  Q  L  A  K  A  I  G  I  E  Q  P  S  L  V  R  T

TTGGATCAACTTGAAGATAAGGGGCTAATTTCGCGGCAAACCTGCGCCAGCGATCGTCGCGCTAAG
L  D  Q  L  E  D  K  G  L  I  S  R  Q  T  C  A  S  D  R  R  A  K

CGGATTAAACTGACCGAAAAAGCGGAGCCGCTGATCGCTGAGATGGAAGAGGTCATTCATAAAACG
R  I  K  L  T  E  K  A  E  P  L  I  A  E  M  E  E  V  I  H  K  T

CGCGGTGAAATTTTGGCTGGGATTTCTTCAGAGGAGATTGAGCTTCTGATTAAACTTGTCGCTAAA
R  G  E  I  L  A  G  I  S  S  E  E  I  E  L  L  I  K  L  V  A  K
```

∇*TnZeo* insertion site for LNWI-SlyA'

```
CTTGAACACAACATTATGGAATTGCATTCTCACGATTGA
L  E  H  N  I  M  E  L  H  S  H  D  Z


LNWI:       1ATGGAATCGCCACTAGGTTCTGATCTGGCACGGTTG⇒ MESPLGSDLARL.....
LNWIΔSlyA: 1ATGGAATCCTGTCTCTTATACACATCTCAACCCTGA⇒ MESCLLYTSQPZ

LNWI:       405AACATTATGGAATTGCATTCTCACGATTGA??????⇒ NIMELHSHDZ
LNWI-SlyA':405AACATTATCTGTCTCTTATACACATCTCAACCCTGA⇒ NICLLYTSQPZ
```

Fig. 2. Nucleotide and deduced amino acid sequences and alignments of the coding regions of *slyA* for LNWI, LNWIΔSlyA, and LNWI-SlyA′. *TnZeo* insertion sites are indicated in bold. Divergences, conferred by *TnZeo*, are underlined.

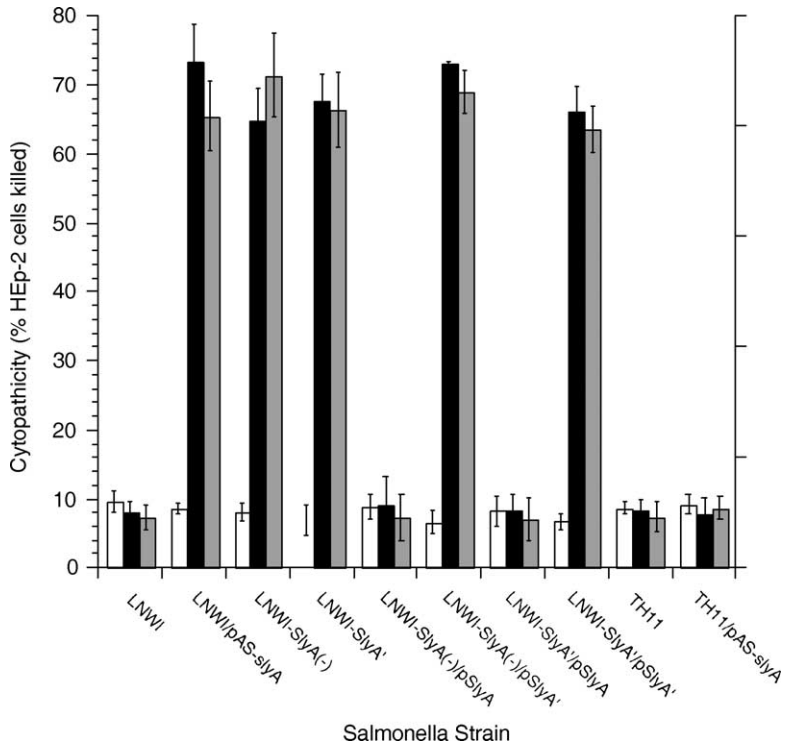

Fig. 3. Evaluation of cytopathicity of LNWI, *TnZeo* insertional mutants of LNWI, and control strains. The ability to damage HEp-2 cells was evaluated for native bacteria (open bars), bacterial lysates (black bars), and supernatants from strains transformed with pBRP (gray bars). LNWI-SlyA(−) is equivalent to LNWIΔSlyA.

with a high copy plasmid encoding SlyA or SlyA′ (pSlyA and pSlyA′, respectively); TH11, an antibiotic sensitive strain of DT104 [6]; and TH11 transformed with pAS-*slyA*.

As shown in the graph of Fig. 3, none of the strains were capable of directly causing cytopathicity in HEp-2 cells. We therefore, hypothesized that SlyA may not be influencing Clg secretion, a problem we encountered in a previous study with *clg* [4], so we additionally assessed cytopathicity in the presence of bacterial lysates. Cytopathicity was additionally evaluated in HEp-2 cells exposed to supernatants from strains transformed with a plasmid (pBRP, MolBioTech) that encodes a lipoprotein (bacteriocin release protein) facilitating cellular leakage of proteins from bacteria [7].

Increases in HEp-2 cell death were observed upon exposure to lysates from LNWI in which *slyA* expression was perturbed either by deletion, truncation, or antisense expression. Similar findings were observed for HEp-2 cells exposed to supernatants from LNWI/pBRP transformants with altered *slyA* expression. SlyA was capable of exerting a dominant phenotype, in regards to repression of cytopathicity, over SlyA′ as observed in LNWI-SlyA′/pSlyA and LNWI/pSlyA′ (latter not shown).

2.3. Extension of the cytopathic phenotype to non-DT104 strains of multiresistant Salmonella

To evaluate the possibility that SGI1 may contribute to the cytopathic phenotype, we assessed cytopathicity in other multiresistant strains of *Salmonella* transformed with pAS-*slyA*. SL1344, a common laboratory strain of *S. typhimurium* that lacks antibiotic resistance [8], was used as an additional control. As shown in Table 3, there was a direct correlation between cytopathic capabilities and SGI1. Specifically, pAS-*slyA* transformation led to a cytopathic phenotype for multiresistant DT120, DT193, and U302 which are three phagetypes of *S. typhimurium* that possess SGI1 [6]. Additionally, multiresistant *S. enterica* serotypes Agona (*S. agona*) and Infantis (*S. infantis*) transformants were also capable of demonstrating the cytopathic phenotype. These strains are multiresistant due to the presence of SGI1 (Refs. [6,9], respectively). The cytopathic phenotype was not observed in *S. typhimurium* DT208, *S. typhimurium* strain 202/37, and *S. enterica* serotype Dublin (*S. dublin*) strain 9276 that are multiresistant but do not possess SGI1 [6]. The high copy antisense plasmids also rendered transformants unable to survive within bovine macrophages.

2.4. Expression of slyA and clg in LNWI and the relationship to cytopathicity and macrophage survival

To evaluate *clg* expression in the absence or presence of SlyA, we performed RT-PCR on the strains used in Fig. 3. Primers for *slyA* were designed to avoid the LNWI-SlyA′-specific truncated region while *clg* primers are those used previously [4]. As shown in Fig. 4, *clg* transcripts were only present in the multiresistant strains when SlyA was absent or when a truncated SlyA was present. The absence of SlyA, via the *slyA* antisense

Table 3
Relationships between cytopathicity, macrophage survival, *slyA* and *clg* transcription, and the presence of SGI1 in various *Salmonella* transformed with pAS-*slyA*

Strain	Cytopathicity[a]	Macrophage survival[b]	Clg/SlyA[c]	SGI1 status
S. dublin/pAS-*slyA*	8.3+1.2	~0	(−)/(−)	(−)
S. agona/pAS-*slyA*	69.5+4.7	~0	(+)/(−)	(+)
S. infantis/pAS-*slyA*	78.3+5.7	~0	(+)/(−)	(+)
795/pAS-*slyA*[d]	71.2+5.1	~0	(+)/(−)	(+)
U302/pAS-*slyA*	77.3+5.6	~0	(+)/(−)	(+)
DT208/pAS-*slyA*	8.3+1.4	~0	(−)/(−)	(−)
DT193/pAS-*slyA*	71.3+3.4	~0	(+)/(−)	(+)
DT120/pAS-*slyA*	72.4+6.1	~0	(+)/(−)	(+)
202/37/pAS-*slyA*[d]	8.1+0.9	~0	(−)/(−)	(−)
SL1344	8.4+1.7	22.7+3.1	(−)/(+)	(−)
SL1344/pAS-*slyA*	7.3+1.2	~0	(−)/(−)	(−)
SL1344/pAS-*slyA*[d]	9.3+1.1	~0	(−)/(−)	(−)
SL1344/pAS-*slyA*[e]	6.9+1.2	1.9+0.3[f]	(−)/(+)[f]	(−)

Transformation utilized the pCR2.1 version of pAS-*slyA* unless stated otherwise.
[a] Percent, given as mean ± SEM, of HEp-2 cells lysed in triplicate experiments; via bacterial lysates.
[b] Percentages of bacteria recovered from bovine macrophages after 16 h; >5% considered as 'survival'.
[c] As determined by RT-PCR.
[d] Transformed with the pCRXL version of pAS-*slyA* (high copy).
[e] Transformed with the pBAD version of pAS-*slyA* (medium copy).
[f] Lower copy number of pBAD allows for some availability (visibility) of *slyA* transcripts and thus some synthesis of SlyA.

plasmid, did not lead to expression of *clg* in the isostrain (TH11) lacking the SGI1. SlyA was capable of exerting a dominant phenotype, in regards to repression of *clg* transcription, over SlyA′.

The lack of *slyA* amplicons for LNWI/pAS-*slyA* and TH11/pAS-*slyA* was not consistent but is shown in this figure. This inconsistency likely reflects the copy number variability (100–300 plasmids per bacterium) of pCR2.1::pAS-*slyA*, i.e. a varying stoichiometric ratio between antisense and sense transcripts, since a *slyA* amplicon could be regularly observed when a medium copy number vector (pBAD, Invitrogen; about 30 copies per bacterium) was used instead of pCR2.1 or pCRXL. Regardless of the copy number, the pAS-*slyA* plasmid was still capable of preventing translation of the *slyA* transcript as evidenced by an inability to survive within bovine macrophages for SL1344 or TH11 each transformed with the pBAD::pAS-*slyA* plasmid (Table 3 for SL1344, data not shown for TH11). The pBAD::pAS-*slyA* experiments were not feasible in the multiresistant strains since pBAD encodes only for ampicillin resistance and all of the multiresistant strains were ampicillin resistant.

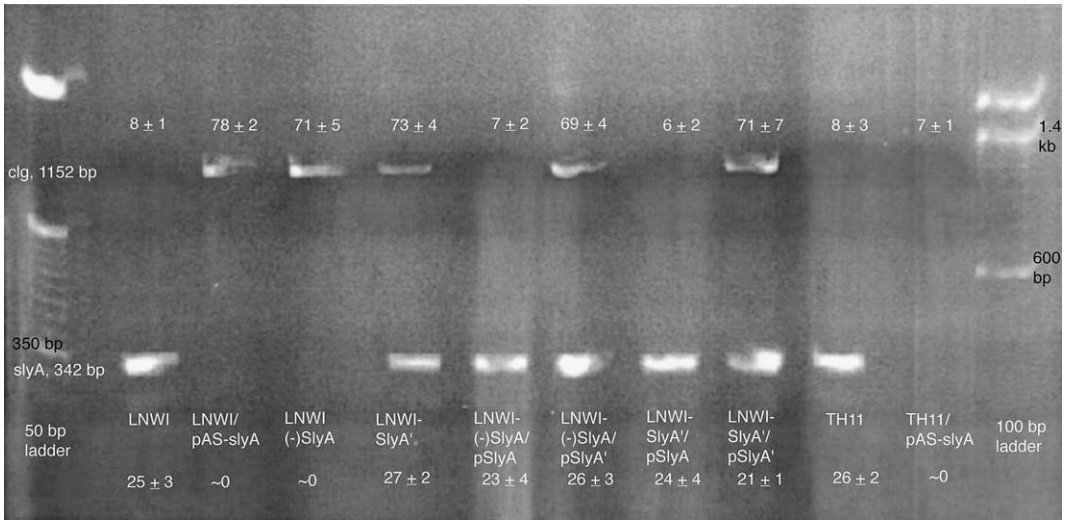

Fig. 4. Relationships between *clg* and *slyA* expression, cytopathicity, and macrophage survival for LNWI, *TnZeo* insertional mutants of LNWI (LNWI-SlyA(−) and LNWI-SlyA′), and control strains. Expression was determined by RT-PCR and the amplicons are shown in an agarose gel stained with ethidium bromide. Percentages of HEp-2 cells lysed, upon exposure to bacterial lysates, are given above the amplicons (or lack thereof) while percentages of bacteria recovered from bovine macrophages are indicated below the amplicons. Molecular weight ladders are shown on the far right (100 bp) and far left (50 bp) lanes with the 350, 600, and 1400 bp markers indicated near their respective molecular weight standards. The size of the expected *clg* and *slyA* transcripts are indicated on the left. LNWI-SlyA(−) is equivalent to LNWIΔSlyA. RT-free assays yielded no amplicons (data not shown).

Since SlyA is involved in conferring intra-macrophage survival to *Salmonella* [10] and since the cytopathic phenotype was observed in cattle, we assessed the ability to survive in bovine macrophages [11]. As shown in the lower portion of Fig. 4, the *slyA* antisense plasmid and the SlyA deletion led to an inability to survive within macrophages. However, the SlyA truncation did not perturb intra-macrophage survival as evidenced by a survival rate equivalent to that of the wild-type strain.

Cytopathicity, determined using bacterial lysates, is indicated by the numbers (% HEp-2 cells dead) above the amplicons in Fig. 4. As previously demonstrated in Fig. 3, cytopathicity was observed in eukaryotic cells exposed to lysates from LNWI in which *slyA* expression was perturbed either by deletion, truncation, or antisense expression.

3. Discussion

The studies presented herein indicate that SlyA represses the expression of *clg*, a gene encoding a collagenase involved in a cytopathic phenotype in DT104 [4]. Additionally, however, derepression is dependent upon elements specific to the SGI1. This island contains multiple antibiotic resistance genes along with 10 unknown ORFs [8] one of which may participate in *clg* derepression.

SlyA is a protein involved in regulating numerous functions in *Salmonella* [12]. Deleting SlyA enabled *clg* expression at the expense of intra-macrophage survival. However, truncating SlyA led to *clg* derepression and maintenance of intra-macrophage survivability. Therefore, it appears possible that carboxyl-terminal cleavage of SlyA could lead to collagenase expression without compromising another virulence phenotype.

It would appear that there are at least five components to the cytopathic phenotype of DT104. The first component, as described previously [4], is *clg*. The second component is *slyA*, a repressor of *clg* expression. The third component is an unknown DT104-specific element, probably present in SGI1, that activates *clg* expression when the SlyA-mediated repression is halted. The fourth component, also unknown at this time, potentially controls SlyA cleavage. The fifth component, yet another unknown factor, regulates Clg secretion. Since *clg* and *slyA* are present in all *Salmonella* and since the DT104-specific activator appears to be constitutive, it is possible that the regulators of SlyA cleavage and Clg secretion may be inducible and that this induction is more likely to occur in veal calves. This is especially possible since the cytopathic phenotype has only been observed clinically in veal calves.

In summary, we have identified SlyA as the repressor of *clg* expression with derepression occurring only in *Salmonella* possessing a specific multiresistance gene island. This study provides the basis for fully elucidating the cytopathic phenotype by identifying mechanisms leading to SlyA cleavage and Clg secretion. Ongoing studies will potentially identify these mechanisms and the relationships to veal calves.

4. Material and methods

4.1. Creation of TnZeo insertional mutants

To create the zeocin-based transposon, we chose the pMOD™-2<MCS> transposon construction vector (Epicentre). The zeocin resistance gene was PCR-amplified, using pCRXL (Invitrogen) as the template, with overhanging *Sal*I and *BamH*I sites engineered onto the forward and reverse primers (provided in Table 2), respectively. Amplicons were digested with *Sal*I and *BamH*I restriction enzymes (New England Biolabs) and cloned into the pMOD multiple cloning site which is flanked by two hyperactive 19-bp mosaic end sequences. The full-length zeocin resistance-bearing transposon (*TnZeo*) was obtained by PCR-amplification using primers (provided in Table 2) which flank the 19-bp mosaic end sequences. Amplicons of *TnZeo* were then incubated with EZ::TM transposase (37 °C for 10 min, without Mg^{2+}), resulting in the EZ::*TnZeo* transposome. The EZ::*TnZeo* transposome was then electrotransformed into LNWI using approximately 10^{10} bacteria and 0.1 μg of transposome in 0.2 cm cuvettes, 2.5 kV and 25 μF in the BioRad GENE PULSER II. Transformants were selected for zeocin resistance (25 μg/ml) and screened for *clg* expression using RT-PCR as previously described [4]. Once the candidates of such were identified, genomic DNA was isolated (G NOME™ DNA Kit, BIO 101), digested with *EcoR*V (New England Biolabs) at 37 °C for 16 h and then self-ligated at 16 °C for 24 h with T_4 DNA ligase. To amplify the DNA fragments containing the *TnZeo* insertion, inverse PCR (primers provided in Table 2) was performed on the self-ligated fragments. Subsequently, the amplicons were agarose gel-purified (QIAEX II, Qiagen), cloned into pCR2.1 (Invitrogen), and transformed into Top10′ cells (Invitrogen). Purified DNA was prepared from the individual transformants and submitted for DNA sequencing.

4.2. RT-PCR experiments and high-throughput screening

RT-PCR was conducted in order to confirm the expression of the *clg* and *slyA* genes. RNA was isolated from cultures grown under anaerobic conditions using the RNeasy Mini Kit as modified per the RNAprotect for Bacteria protocol, utilizing enzymatic lysis for the initial cell wall disruption (Qiagen). RT-PCR was carried out using the SuperScript One-Step RT-PCR with Platinum *Taq* kit (Invitrogen), using either the *clg* or *slyA* primers described in Table 2, under the conditions specified by the manufacturer. PCR conditions were as follows: 35 cycles of 94° for 15 s, 55° for 30 s, and 72° for 1 min, followed by 72° for 5 min. Reactions were

visualized on 1.5% agarose gels. The *slyA* amplicon is 342 bp while the *clg* amplicon is 1152 bp.

High-throughput screening for *clg* transcripts was performed by examining the presence of *clg* expression, using RT-PCR and agarose gels, in pools of LNWI-*TnZeo* insertion mutants via a checkerboard approach [13]. Briefly, LNWI-*TnZeo* insertion mutants were pooled and approximately 2×10^8 insertion mutants were placed in individual wells of 96-well microtiter plates (i.e. 1.92×10^{10} insertion mutants per plate). Twenty subpools, consisting of 12 'column' pools and eight 'row' pools, were then created and RT-PCR was performed using these subpools thus allowing 96 wells to be screened in 20 RT-PCR reactions. Individual colonies from cross-matched (e.g. rowA/column4) RT-PCR positive pools were then isolated and subjected to the RT-PCR procedure until an RT-PCR positive clone was identified.

4.3. Cytopathicity experiments

Cytopathicity was evaluated by enumerating live versus dead HEp-2 cells using 0.4% trypan blue. HEp-2 cells were incubated with whole bacteria or bacterial supernatants or bacterial lysates, derived as previously described [4], for 1 h. Approximately 100 cells were counted for each well of tissue culture cells. Assays were performed in triplicate.

4.4. Creation of the slyA- and slyA′-bearing plasmids

The *slyA* antisense (pAS-*slyA*) and sense (pSlyA) plasmids were created by PCR-based cloning of the *slyA* gene from LNWI. The full-length gene was ligated into pCR2.1 (or pCRXL for kanamycin resistant *S. typhimurium* strains 795 and 202/37; or pBAD additionally for SL1344 and TH11; all three vectors from Invitrogen) and a clone with *slyA* in the antisense orientation became pAS-*slyA* while a clone with *slyA* in the sense orientation became pSlyA. The pSlyA′ plasmid was created in a similar manner using LNWI-SlyA′ as the source of template DNA with a *TnZeo* insertion-specific primer used as the reverse primer. These plasmids were then amplified in a recombinase-free *Salmonella* [14] and then transformed into various *Salmonella* strains. Transformants were selected using 50 μg/ml kanamycin (Sigma) or 50 μg/ml zeocin (Invitrogen).

4.5. Macrophage survival assays

Macrophage survival was assessed in triplicate using bovine macrophages [11] and the protocol described previously [15]. However, given the multiresistance of LNWI and other strains described herein, gentamicin was replaced with high concentration florfeniol (300 μg/ml) in the extracellular lysis phase as previously described [16].

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